FGF upregulates VEGF via MAPK Signaling Pathway in Human Airway Smooth Muscle cells

Anna Willems-Widyastuti[1], Bart M. Vanaudenaerde[1], Robin Vos[1], Ellen Dillissen[1], Stijn E. Verleden[1], Stéphanie I. De Vleeschauwer[1], Annemie Vaneylen[1], Willem I. de Boer[2], Hari S. Sharma[3] and Geert M. Verleden[1]

[1]Laboratory of Pneumology, Katholieke Universiteit Leuven, Leuven, Belgium;

[2]Department of Pulmonology, LUMC, Leiden, The Netherlands;

[3] Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

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Address for correspondence: Prof. Dr. G. Verleden

University Hospital Gasthuisberg

Lung Transplantation Unit

49 Herestraat, B-3000 Leuven, Belgium

Tel: + 32 16 346800 Fax: + 32 16 346803

E-mail: **geert.verleden@uzleuven.be**

ABSTRACT

Fibroblast growth factors, FGF-1, FGF-2 and vascular endothelial growth factor (VEGF) are elevated in chronic inflamed airways. Airway smooth muscle (ASM) cells are known to synthesize VEGF. We investigated the contribution of FGF-1/-2 on the VEGF production in ASM cells, the involvement of mitogen-activated protein kinases (MAPK) and the modulatory effects of azithromycin and dexamethasone.

Human ASM cells were treated with 10ng/ml FGF-1 or FGF-2. Specific blockers for ERK1/2^{MAPK} (U0126), p38^{MAPK} (SB239063), JNK (curcumin), dexamethasone or azithromycin were added 30 minutes prior to stimulation. Expression of VEGF (VEGF-A, $VEGF_{121}$ and $VEGF_{165}$) was assessed by quantitative PCR, VEGF release by ELISA and MAPK phosphorylation by Western blotting.

FGF-1/-2 upregulated mRNA expression of VEGF (VEGF-A, $VEGF_{121}$ and $VEGF_{165}$) and its release by 1.8 fold (FGF-1) and 5.5 fold (FGF-2). Transient increase in ERK1/2^{MAPK} and p38^{MAPK} phosphorylation and subsequent release of VEGF from FGF-1/-2 treated human ASM cells was inhibited by respective blockers. Furthermore, both dexamethasone and azithromycin reduced the VEGF secretion mediated by the p38^{MAPK} pathway.

In conclusion, we demonstrate that FGF-1 and FGF-2 upregulate VEGF production via ERK1/2^{MAPK} and p38^{MAPK} pathways. The anti-angiogeneic effect of dexamethasone and azithromycin may potentially contribute to tackle VEGF-mediated vascular remodelling in chronic airway diseases.

Keywords: Airway Smooth Muscle Cell, Angiogenesis, Azithromycin, Fibroblast Growth Factor, Mitogen-Activated Protein Kinases, Vascular Endothelial Growth Factor

INTRODUCTION

Chronic airway diseases such as asthma and COPD are characterized by expiratory airflow limitation due to thickening of the airway wall, repeated epithelial injury and a mixture of inflammatory exudates with excessive mucus secretion (1). Thickening of the airway wall is mostly caused by airway smooth muscle (ASM) cells hypertrophy and hyperplasia and subepithelial fibrosis (2), highlighting a pivotal role of ASM cells as effector cells during chronic airway inflammation (3;4). ASM cells are capable to synthesize and release a variety of extracellular matrix proteins that may perpetuate the inflammation and airway remodelling in atopic asthma and COPD (5;6). During chronic airway inflammation, fibroblast growth factor (FGF-2), a potent fibroproliferative factor is increased in the broncho-alveolar lavage (BAL) fluid of asthmatics (7). Furthermore, Kranenburg *et al* showed a positive correlation of FGF-1, FGF-2 and FGF receptor-1 (FGFR-1) expression in the bronchial epithelium and ASM cells with the severity of COPD indicating that the FGF-FGFR system contributes to the pathogenesis of chronic airway diseases (8).

Angiogenesis and deregulated bronchial vasculature are prominent in the structurally altered and inflamed airways which further contribute to the severity of chronic airway diseases (9). Vascular endothelial growth factor (VEGF), a homodimeric glycoprotein and endothelial cell specific mitogen, plays a key role in physiological and pathophysiological angiogenesis. There are at least five VEGF gene products termed as A to E of which VEGF-A (here after mentioned as VEGF) is a key regulator of angiogenesis and vascular permeability (10). Different isoforms of VEGF ($VEGF_{121, 165, 189, 206}$) due to alternate splicing of its mRNA have been reported (10). Increased VEGF levels are observed in BAL fluid, sputum and bronchial biopsies of asthma and COPD patients (9;11-13). Human ASM cells constitutively express VEGF and its isoforms of which $VEGF_{121}$ is the dominant one (14).

Airway structural changes are associated with bronchial angiogenesis and vascular remodelling of which the molecular mechanisms are not fully understood. We and others have shown that ASM cells express and release several growth factors *in vitro* in response to a number of stimuli (14;15). In view of the increased bronchial levels of FGFs and VEGF in patients with chronic airway diseases, we hypothesize that the FGF-FGFR-1 system contributes to angiogenesis and vascular remodelling via VEGF. An *in vitro* study was established using primary cultures of human ASM cells treated with FGF-1 or FGF-2 and assessing the VEGF production both at mRNA and protein level. Furthermore, we investigated the involvement of the mitogen-activated protein kinase (MAPK) pathway in the production of VEGF in FGF treated ASM cells.

Currently, the combination of inhaled corticosteroid and long-acting β-adrenergic agonists is the main treatment modality for chronic airway diseases (16). Unfortunately, this treatment is less effective in severe asthmatic and smoking related diseases such as COPD (16;17). Treatment with macrolides is moving into the clinical practice of chronic lung disorders (18), and many studies already addressed their anti-inflammatory effect (19). Therefore, we explored the effects of the corticosteroid dexamethasone and the macrolide azithromycin on the release of VEGF by human ASM cells treated with FGFs and assessed its underlying signaling mechanisms *in vitro*.

MATERIALS AND METHODS

This study has been approved by the local medical ethical committee of the KUL (S51577) and the biosafety committee MS20101570

Isolation and culture of human ASM cells

Primary human ASM cells were isolated from trachea obtained from different donors (n=3 to 6) for lung transplantation. Dissected smooth muscles were cut into small pieces and cultured

in DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (1,25 µg/ml) (Invitrogen, Merelbeke, Belgium), further maintained at 37°C in a humidified atmosphere of 5% CO_2 as previously described (15). At confluence, ASM cells were verified for a spindle shaped morphology and "hill and valley pattern" by light microscopy and further characterized by immunocytochemistry for α-smooth muscle actin, smooth muscle myosin heavy chain (SM1 and SM2) and calponin (Sigma-Aldrich, Bornem, Belgium). Confluent cells in the fourth to sixth passage were used for all experiments.

Incubation of human ASM cells with FGFs

ASM cells were seeded in 12 well plates or in T25 cm^2 culture flasks and grown for 48 hours in DMEM before further grown in serum-deprived medium (DMEM containing 0.2% FBS) for 72 hours. Growth-arrested ASM cells were treated with 10ng/ml of FGF-1 or FGF-2 (Sigma-Aldrich) in fresh serum-deprived medium. To verify the involvement of MAPK signaling pathway in VEGF release by ASM cells, the MAPK specific inhibitors for ERK $1/2^{MAPK}$: U0126 (10 µM), $p38^{MAPK}$: SB239063 (10 µM) or JNK: curcumin (1 µM)(VWR; Haasrode, Belgium)were added 30 minutes prior to FGF treatment. To examine the possible role of dexamethasone (Sigma) and azithromycin (Sigma), ASM cells were pre-treated with 1 µM dexamethasone or 10µM azithromycin 30 minutes prior to FGF incubation. Cell-conditioned medium of each experiment was collected; cells were harvested in lyses buffer and stored at -80°C

VEGF mRNA quantification by real-time PCR

ASM cells were treated with 10ng/ml FGF-1 or FGF-2 for 1, 2, 4, 6 or 18 hours. Total cellular RNA was isolated using PureLink total RNA purification system (Invitrogen, Merelbeke,

Belgium). An on-column DNase digestion was incorporated into the procedure to remove any contaminating genomic DNA. Total RNA concentration was estimated by optical density measurements and an A_{260}/A_{280} ratio of ≥ 1.8 was accepted. One µg of total RNA was used to generate cDNA using Ready-to-go T-primed First Strand Kit (GE Healthcare, Roosendaal, The Netherlands). Transcript levels were determined by real time PCR employing ABI prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA) and using one step SYBR Green I PCR Master Mix Reagent Kit (Eurogentec, Herstal, Belgium). All oligonucleotide primers were purchased from Eurogentec (20). Each primer was used at a concentration of 0.3 µM. The thermal cycler profile consisted of a 2-min step at 45°C, 10-min of Taq-polymerase activation at 95°C, followed by 40 cycles of PCR at 95°C for 15-sec and 60°C for 1-min. The results were quantified using comparative threshold (*Ct)* method. The *Ct* values of VEGF mRNA were normalized to *Ct* value of GAPDH mRNA, and presented as relative *Ct* value of VEGF mRNA expression. The relative *Ct* values of stimulated ASM cells to control (not-stimulated cells) were calculated using $2^{-\Delta\Delta Ct}$ formula as a fold change (21).

VEGF protein measurement by ELISA

Monolayer growth-arrested human ASM cells were incubated with 10ng/ml of FGF-1 or FGF-2 for 24 hours. The involvement of MAPK pathways in the secretion of VEGF was assessed by treating cells with U0126 (10 µM), SB239063 (10 µM) or curcumin (1 µM) for 30 minutes prior to FGF stimulation. Similarly, the effect of dexamethasone (1 µM) or azithromycin (1 µM or 10 µM) on the release of VEGF were examined. VEGF protein concentrations were determined in 100 µl undiluted conditioned medium using a sandwich ELISA method according to the manufacturer's instructions (R&D System Europe Ltd, Abingdon, UK). The concentration of VEGF was expressed in pg/ml, with a detection limit of 5 pg/ml.

Quantification of MAPK activation by western blotting

To determine the activation of MAPK pathway in response to FGF treatment, growth-arrested ASM cells were treated with 10 ng/ml of FGF-1 or FGF-2 for short periods (5, 10, 15, 30 or 60 minutes). To examine the effects of specific inhibitors U0126, SB239063 or curcumin on MAPK activation, cells were treated with specific inhibitor 30 minutes prior to FGF stimulation. The effects of anti-inflammatory drugs were studied on cells pre-treated with dexamethasone (1 μM) or azithromycin (10 μM) for 30 minutes prior to FGF incubation for 30 minutes. Cells were washed twice with ice cold PBS before extraction of total protein using cell extraction buffer (Invitrogen) containing a cocktail of protease and phosphatase inhibitors (Complete/ PhosSTOP; Roche, Mannheim, Germany). The protein concentration was determined using a commercially available Bio-Rad protein assay kit (Bio-Rad; Nazareth, Belgium). Protein extracts (50 μg) were fractionated by SDS-polyacrylamide gelelectrophoresis and transferred onto a PVDF membrane (GE Healthcare; Roosendaal, The Netherlands). The membranes were incubated for 1 hour with blocking agent (GE Healthcare) and consecutively overnight at 4°C with following primary antibodies: anti-total or anti-phosphorylated $p38^{MAPK}$, ERK $1/2^{MAPK}$ or JNK (Bioke; Leiden, the Netherland). After several washing steps, the target proteins were detected by the ECL western blotting detection system and ECL hyperfilm (GE Healthcare) according to the manufacturer's instruction. Activation levels were expressed as the ratio of phosphorylated to non-phosphorylated signaling protein.

Statistics

The results were expressed as mean ± standard error of the mean (SEM) from 4-6 different experiments. Student's *t*-test and Mann-Whitney U test was used for protein experiments (ELISA and Western Blotting) whereas one way ANOVA combined with Bonferroni test was employed for mRNA experiments (real-time PCR). Graphpad Prism 4.0 (San Diego, CA,

USA) was used for data analysis. Differences were considered statistically significant when $p<0.05$.

RESULTS

The effects of FGFs on the VEGF expression and secretion in human ASM cells

Both FGF-1 and FGF-2 augmented VEGF mRNA expression in human ASM cells. FGF-1 induced the expression of VEGF-A, $VEGF_{121}$ and $VEGF_{165}$ in transient manner reaching maximal levels at 2h (n=5, $p<0.001$) and thereafter mRNA levels declined (figure 1A). However, FGF-2 induced VEGF mRNA expression as early as 2h, and this remained elevated up to 6h (n=5, $p<0.001$) and then the levels declined (figure 1B).

In analogy to the mRNA expression, FGF-1 as well as FGF-2 significantly induced VEGF protein release after 24 h of stimulation (figure 1C). FGF-1 induced a 1.84 ±0.05 fold increase of VEGF production by ASM cells (n=5, $p=0.0079$) as compared to control whereas, FGF-2 increased the VEGF protein secretion by 5.49±0.52 fold (n=5, $p=0.0079$) after 24 h of stimulation (Figure 1C).

The activation of the MAPK pathways by FGFs

Data shown in figure 2 demonstrated a rapid and robust phosphorylation of all three MAP kinases namely, $ERK1/2^{MAPK}$, $p38^{MAPK}$ and JNK 5 min before the levels declined gradually. Though levels declined, both FGF-1 and FGF-2 induced phosphorylation of $ERK1/2^{MAPK}$ remained significantly elevated even after 60 min of incubation as compared to respective controls.

The effects of MAPK inhibitors and azithromycin on MAPK activation

U0126 significantly reduced the rapid phosphorylation of $ERK1/2^{MAPK}$ in response to FGF-1 (Figure 3A, $p=0.0088$) as well as by FGF-2 (Figure 3B, $p=0.0032$). Similarly, when cells were

pretreated with SB239063, a significantly reduced $p38^{MAPK}$ activation was observed in FGF-1 (Figure 3C, p=0.0254) and FGF-2 (Figure 3D) treated human ASM cells. Pre-treatment with curcumin did not reduce FGFs induced JNK phosphorylation, despite rapid activation of this pathway in response to both FGF-1 and FGF-2 treatment (Figure 3E and 3F, respectively).

The effect of azithromycin and dexamethasone on the MAPK pathway

Pre-treatment with azithromycin or dexamethasone revealed no reduction in FGFs stimulated $ERK1/2^{MAPK}$ phosphorylation. When cells were pretreated with azithromycin, a significant reduction in $p38^{MAPK}$ activation was observed in both FGF-1 (Figure 3C, p=0.0236) as well as FGF-2 (Figure 3D, p=0.0323) stimulated human ASM cells. Dexamethasone pretreatment resulted in drastic inhibition of $p38^{MAPK}$ activation in FGF-1 (p=0.0494) or FGF-2 (p=0.0261) stimulated cells whereas, $ERK1/2^{MAPK}$ and JNK pathway remained unaffected. Interestingly, azithromycin also reduced JNK activation in response to FGF-2 treatment in ASM cells (Figure 3F, p=0.0244).

The effects of MAPK inhibitors on the VEGF release

A 24h of incubation with SB239063 resulted in significantly reduced levels of VEGF in conditioned media derived from FGF-1 (Figure 4A, p=0.0177) or FGF-2 (Figure 4B, p=0.0225) treated human ASM cells. Co-incubation of U0126 caused a significant attenuation of VEGF protein release either in FGF-1 (Figure 4A, p=0.0257) or in FGF-2 (Figure 4B, p=0.0051) stimulated human ASM cells. Curcumin could not reduce the VEGF protein release by FGF-1 or FGF-2-stimulated ASM cells (n=4, p=0.4176 and n=6, p=0.4075 respectively).

The effect of azithromycin or dexamethasone on the VEGF release

A reduction in VEGF protein release by 10 μM azithromycin was detected in FGF-1 (Figure 5A, p=0.0081) as well as FGF-2 (Figure 5B, p=0.0011) treated cells after 24h. Dexamethasone (1 μM) treatment drastically decreased the VEGF protein release by FGF-1 or FGF-2 stimulated human ASM cells (Figure 5A, p=0.0113 and Figure 5B, p<0.0001 respectively)**.** ASM cells were also treated for 24 h only with dexamethasone or azithromycin and the toxicity tests revealed that both drugs were not toxic to the cells (data not shown).

DISCUSSION

In this study, we demonstrated that FGF-1 and FGF-2 stimulation of ASM cells *in vitro* leads to: (i) increased expression of VEGF mRNA and its splice variants ($VEGF_{121}$ and $VEGF_{165}$) in a time dependent manner; (ii) increased release of VEGF protein and that FGF-2 is more potent than FGF-1; (iii) activation of MAPK signaling pathways (ERK1/2^{MAPK} and p38^{MAPK}) which mediate VEGF expression and release; (iv) p38^{MAPK} signaling pathway mediated VEGF protein release from human ASM cells that is blocked by the anti-inflammatory drugs azithromycin and dexamethasone.

FGF-1 and FGF-2 are prototypes of the FGF family whereas an increase in their expression has been reported in several pathophysiological conditions (7;8;22). Shute *et al* localized the bronchial FGF-heparan sulphate (FGF-HS) complex on endothelial and in bronchial epithelial basement membrane of asthmatic airways (22). Due to lack of secreting peptide, the release of biologically active FGF can occur by proteolytic degradation, during inflammatory processes or tissue damage (22). In this study, we attempted to mimic the pathophysiological condition of the increased FGF-1 and FGF-2 in chronic inflamed airways by investigating the role of

FGFs in modulating the production of angiogenic molecule, VEGF in human ASM cells *in vitro.*

Having demonstrated that FGF-1/-2 induced VEGF production both at transcriptional as well as at translational level, our data showed the possible contribution of FGF-1 and FGF-2 in angiogenesis and vascular remodeling, a hallmark in chronic lung diseases (9;10). The involvement of VEGF in the pathogenesis of asthma is well documented (9;11;23). In the case of COPD, the expression of VEGF is associated with the development of the disease (9). Kanazawa *et al* reported that differences in VEGF expressions were significantly involved in the patho-physiology of the two major phenotypes of COPD, namely emphysema and chronic bronchitis (24). Chronic bronchitis was suspected to be the overlapping phase between the chronic inflammatory condition of asthma and an early stage of COPD (25).

Santos *et al* reported the enhanced expression of VEGF in the pulmonary arteries of smokers and patients with moderate COPD (26), reflecting the possible effect of cigarette smoke on the increase of VEGF. The study of Rovina *et al* has confirmed that cigarette smoke significantly increases the VEGF level in the airways of both asymptomatic and bronchitis type COPD smokers (13). In a detailed study, Kranenburg and colleagues measured the cellular expression pattern of VEGF and its receptors, Flt-1 and KDR/Flk-1 in central and peripheral airways obtained from (ex)smokers, with or without COPD, and they showed that COPD is associated with increased expression of VEGF in bronchial, bronchiolar and alveolar epithelium, in bronchiolar macrophages, and also in airway and vascular smooth muscle (27).

It is conceivable that cigarette smoke may, at least partly, be responsible for the increase in bronchial FGF-1 and FGF-2 expressions by provoking repeated episodes of injury and inflammatory processes leading to tissue remodeling in current/ex-smokers with COPD (8). In this study, we used human ASM cells derived from (healthy) donors. Our data showed that FGF-1 and FGF-2 are at least in part responsible for increased levels of VEGF in chronic

inflamed airways. Therefore, our results support a pathophysiological evidence for the involvement of VEGF in the development of COPD.

The MAPK pathways, a family of serine-threonine proteins, are known to be involved in ASM cell proliferation in response to a variety of growth factors including TGF-β and FGF (28). We demonstrated that the incubation of human ASM cells with FGF-1 or FGF-2 leads to the rapid and transient activation of MAPK pathways (ERK1/2^{MAPK}, p38^{MAPK} and JNK). Furthermore, we found that ERK1/2^{MAPK} and p38^{MAPK} but not JNK signaling pathway mediates the production of VEGF after FGF-1/-2 treatment of human ASM cells.

Keeping in mind the role of angiogenesis in chronic airway inflammation, we examined whether anti-inflammatory drugs such as dexamethasone and azithromycin may interrupt the release of VEGF protein from FGF-1/-2 stimulated ASM cells and whether MAPK signaling pathways were also involved in this process. Our data showed that dexamethasone and azithromycin individually decrease VEGF release from human ASM cells in response to FGF-1/-2 treatments. Their inhibitory effects were found to be mediated by p38^{MAPK} in particular. Our findings are in agreement with the work reported by Clark *et al* (29) on the p38^{MAPK} pathway deactivation by dexamethasone. In our study, dexamethasone, effectively inhibited both the p38^{MAPK} activation and the secretion of VEGF from human ASM cells *in vitro*.

In addition to their antimicrobial activity, the immunomodulatory effect of macrolides like azithromycin to treat chronic airway diseases gained lots of interest in recent years (19), although the underlying mechanisms of its immunomodulation are still not clear. Interestingly, we also observed that, in response to FGF-2 (but not FGF-1), reduction of the VEGF release from ASM cells correlated with inhibition of JNK signaling pathway. These findings suggest for a potential effect of azithromycin as an anti-angiogenic and immunomodulatory agent at least driven by ASM cells. Due to its known side-effects, long-

term treatment with corticosteroids is not advised. Since azithromycin shares the same signaling pathway as dexamethasone in exerting its inhibitory effect on the VEGF release, the combined treatment of both could allow a lower dosage of corticosteroids and hence decrease its side-effects. On the other hand, azithromycin or macrolides in general could enhance antimicrobial resistance (19). Therefore, more studies are needed to investigate the safe and effective ways of applying this potential drug.

Taken together, our findings suggest that FGF-1 and FGF-2, in addition to their role as extracellular matrix regulators, also function as pro-angiogenic growth factors that could play an important role in bronchial vascular remodeling via VEGF-mediated pathways. The anti-inflammatory drugs, dexamethasone and azithromycin exert inhibitory effects on VEGF release in FGF-treated human ASM cells *in vitro*, thereby, introducing a clinical beneficial contribution of azithromycin and dexamethasone or in combination, to anti-angiogenic events in chronic inflammatory airway diseases.

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FIGURES:

Figure 1A

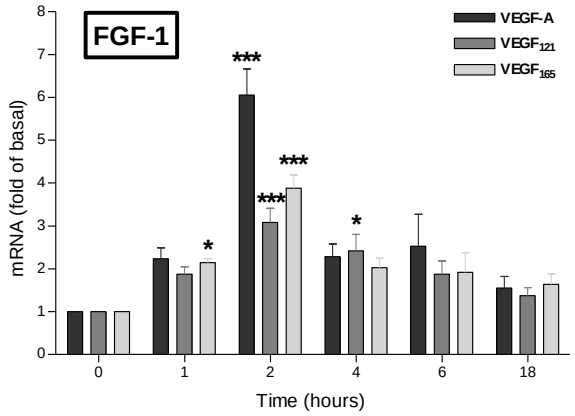


Figure 1B

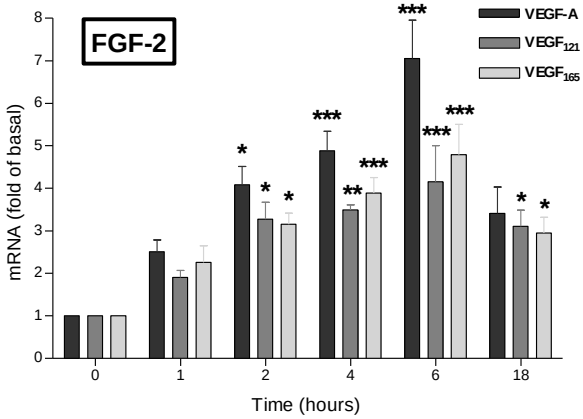


Figure 1C

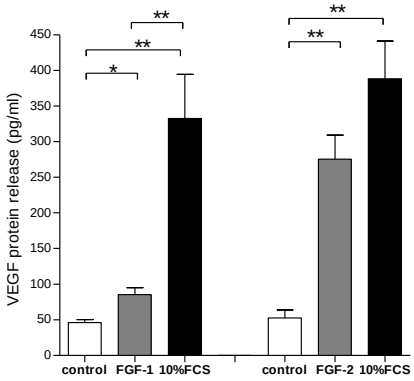


Figure 2

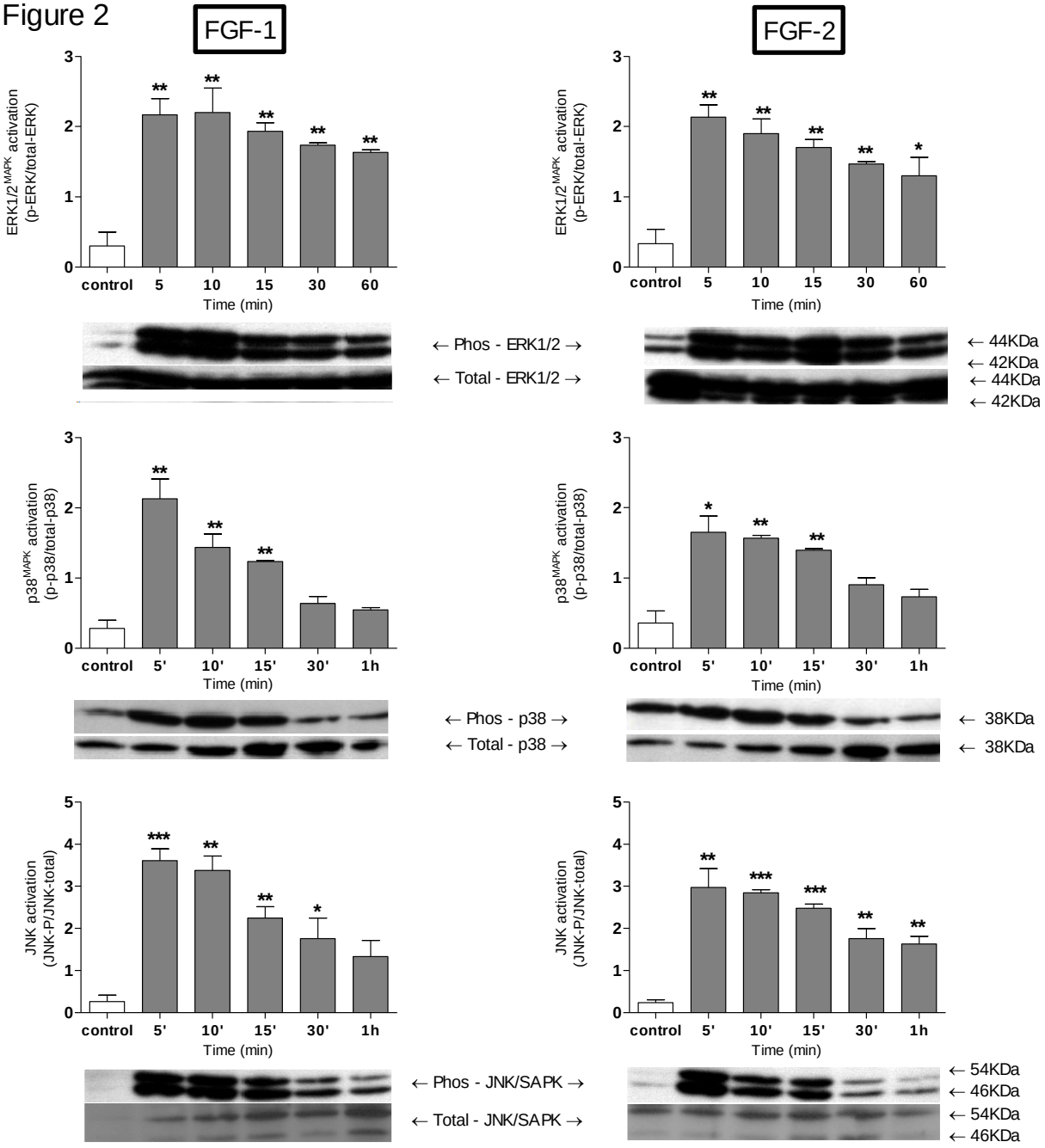

FGF-1
FGF-2
ERK1/2MAPK activation (p-ERK/total-ERK)
control 5 10 15 30 60
Time (min)
← Phos - ERK1/2 →
← Total - ERK1/2 →
← 44KDa
← 42KDa
← 44KDa
← 42KDa
p38MAPK activation (p-p38/total-p38)
control 5' 10' 15' 30' 1h
Time (min)
← Phos - p38 →
← Total - p38 →
← 38KDa
← 38KDa
JNK activation (JNK-P/JNK-total)
control 5' 10' 15' 30' 1h
Time (min)
← Phos - JNK/SAPK →
← Total - JNK/SAPK →
← 54KDa
← 46KDa
← 54KDa
← 46KDa

Figure 3

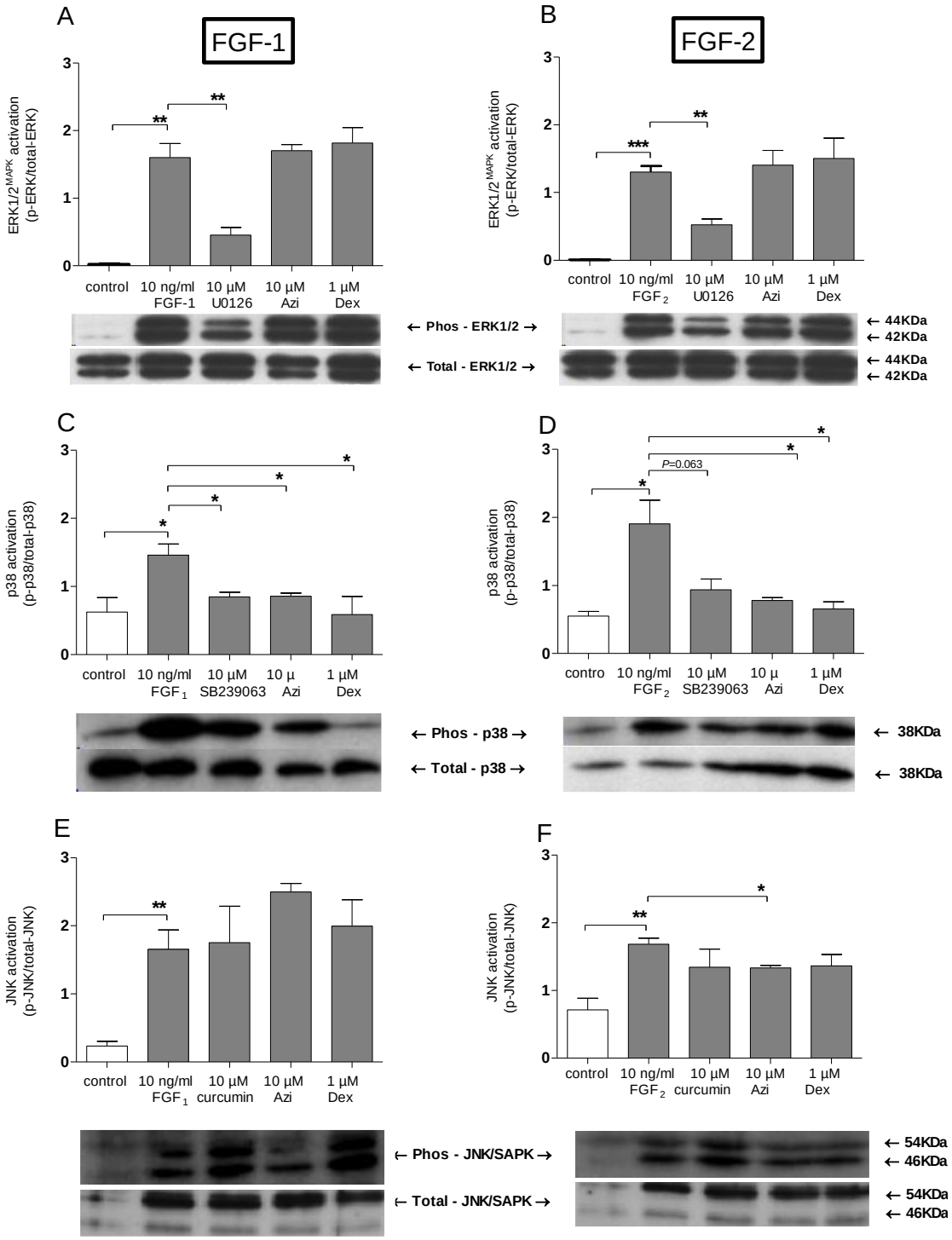

A
FGF-1
B
FGF-2
ERK1/2 MAPK activation (p-ERK/total-ERK)
control
10 ng/ml FGF-1
10 µM U0126
10 µM Azi
1 µM Dex
10 ng/ml FGF2
← Phos - ERK1/2 →
← Total - ERK1/2 →
← 44KDa
← 42KDa
C
D
p38 activation (p-p38/total-p38)
10 ng/ml FGF1
10 µM SB239063
10 µ Azi
1 µM Dex
P=0.063
← Phos - p38 →
← Total - p38 →
← 38KDa
E
F
JNK activation (p-JNK/total-JNK)
10 µM curcumin
← Phos - JNK/SAPK →
← Total - JNK/SAPK →
← 54KDa
← 46KDa

Figure 4

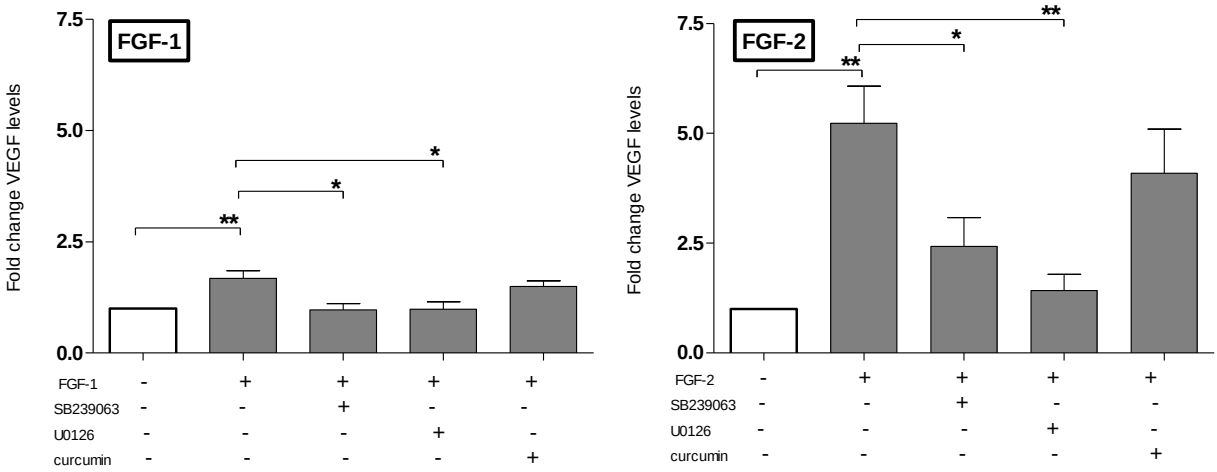
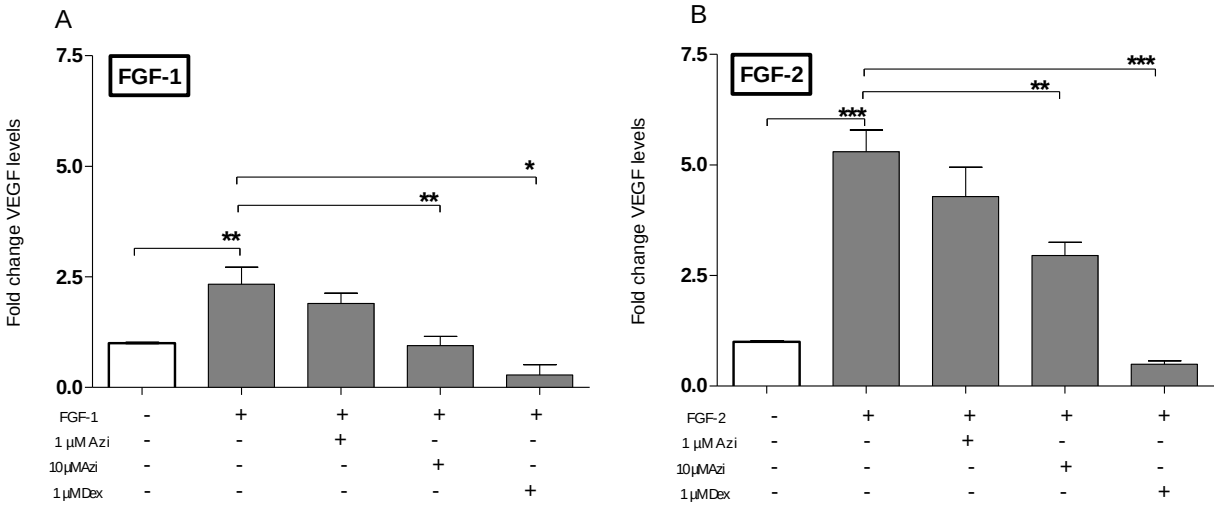


Figure 5



LEGENDS:

Fig.1A and B: VEGF mRNA expression and its splice variants $VEGF_{121}$ and $VEGF_{165}$ by human ASM cells that were either untreated (0h as basal level) and treated with 10 ng/ml of FGF-1 or FGF-2. Results are means±SEM of duplicate measurements representative from 5 human ASM donors and are normalized to GAPDH expressions. Results are expressed relative to VEGF expression in control cells compared with the basal level.*p<0.05; **p<0.01 and ***p<0.001. **Fig.1C:** FGF-1 or FGF-2 significantly increased VEGF protein release by human ASM cells after 24 h. Compared to untreated cells (control), FGF-1 increased VEGF release by ASM cells (46,42±4,45 vs 85,42±9,75 pg/ml; n=5 p=0,0159), whereas FGF-2 induced more on the VEGF secretion (52,48±11,49 vs 275.60±33.48 pg/ml; n=5 p=0.0079).

Fig.2A-F In response to FGF-1 or FGF-2 treatment, the $ERK1/2^{MAPK}$, $p38^{MAPK}$ or JNK pathway in human ASM cells was activated as early as 5 minutes. Control represented the non-treated cells. Results are shown as diagram and blots. The bar diagram depict the activation considered to the ratio between the phosphorylated per total. Values are shown as mean±SEM of independent experiments from 3 human ASM donors. *p<0.05, **p<0.01 and ***p<0.001.

Fig.3A-F: Activation of $ERK1/2^{MAPK}$, $p38^{MAPK}$ or JNK pathway in response to FGF-1 or FGF-2 treatment in the presence of U0126, SB239063, curcumin or anti-inflammatory drugs dexamethasone and azithromycin was examined by western blotting. Control represented the non-treated cells. Results are shown as diagram and blots. The bar diagram depict the activation considered to the ratio between the phosphorilated per total. Data

represents the mean ± SEM of independent experiments from 3 ASM donors. *p<0.05, **p<0.003

Fig.4: Release of VEGF protein by 10 ng/ml of FGF-1 or FGF-2 –stimulated human ASM cells, either in the absence (control) or presence of MAPK inhibitors: 10 µM SB239063 ($p38^{MAPK}$), 10 µM U0126 ($ERK1/2^{MAPK}$) and 1 µM curcumin (JNK). Data represents the mean±SEM of independent experiments from 4-6 ASM donors (FGF-1 and FGF-2 respectively). Data are expressed as fold change of VEGF release compared to their respective controls. *p<0.05 and **p<0.01

Fig.5: Human ASM cells were stimulated with 10 ng/ml of FGF-1 or FGF-2 for 24 hours, either in the absence or presence of azithromycin (1 µM; 10 µM) or dexamethasone (1 µM). Results represent the mean±SEM of duplicate measurements representative from 3 ASM cells donors. Data are expressed as fold change of VEGF release compared to respective controls. *p<0.05, **p<0.01 and ***p<0.001